Histone Phosphorylation: Stimulation by Adenosine 3′,5′-Monophosphate

Abstract. *Adenosine cyclic 3′,5′-monophosphate at a concentration of 10^{-7}M causes a four- to sixfold increase in the rate of histone phosphorylation catalyzed by a liver enzyme preparation. This observation suggests a mechanism for the induction of RNA synthesis by those hormones that cause increases in the concentration of cyclic AMP.*

Liver extracts contain a protein kinase that catalyzes the transfer of phosphate from adenosine triphosphate (ATP) to serine residues of histones and protamines (*1*). Sutherland (*2*), Krebs and Fischer (*3*), and Krebs (*4, 5*) and their associates have established that a sequence of protein phosphorylation reactions involved in the hormone-induced activation of glycogen phosphorylase is stimulated by adenosine 3′,5′-monophosphate (cyclic AMP). A large number of other hormone responses are mediated by cyclic AMP (*6*), including some which are blocked by inhibitors of protein (*7*) and RNA (*8*) synthesis, but the mechanism by which the cyclic nucleotide acts in these cases is unknown. A cyclic AMP requirement has been observed in a protein kinase reaction more closely related to the one under study in this laboratory. This observation was made with an enzyme, obtained from skeletal muscle by Walsh, Perkins, and Krebs (*5*), which catalyzes a cyclic AMP-dependent phosphorylation of phosphorylase *b* kinase. In collaboration with G. H. Dixon and B. Jergil, University of British Columbia, Walsh *et al.* (*5*) found that the skeletal muscle kinase also catalyzes a cyclic AMP-dependent phosphorylation of protamine. In addition, the activity of a protamine kinase obtained from trout testes (*9*) is increased 20 to 30 percent by cyclic AMP (*10*).

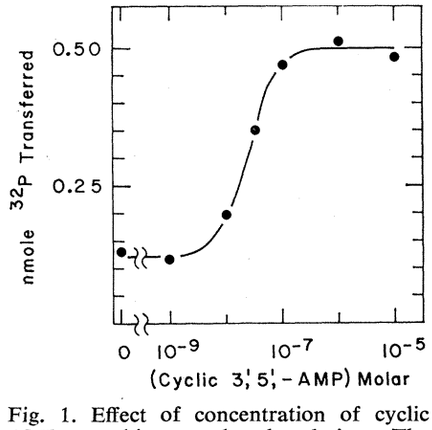

Fig. 1. Effect of concentration of cyclic AMP on histone phosphorylation. The amount of histone kinase present was 2.6 μg, and the specific activity of the ^{32}P-ATP was 1710 count/min per nanomole of β,γ-phosphate. The substrate was f1 histone. Incubation time, 5 minutes. In this experiment, acid-insoluble ^{32}P was counted directly after the washed Millipore filters were glued to planchets.

Prompted by these findings, we conducted tests for the effect of cyclic AMP on the liver kinase preparations which act on histones and protamines. There was no effect of the cyclic nucleotide on protamine phosphorylation. However, phosphorylation of histones by these preparations is stimulated by cyclic AMP.

The histone kinase preparations were purified approximately 150-fold by the following procedure (all operations at 0° to 4°C). "Acetone powder" (24 g) from calf liver (*11*) was ground in a mortar with 300 ml of 0.2*M* $(NH_4)_2SO_4$, 0.05*M* in tris[tris(hydroxymethyl) aminomethane] buffer. (Except where indicated, the *p*H of all buffers used in the procedure was 7.5.) The resulting suspension was stirred slowly for 30 minutes and centrifuged for 20 minutes at 33,000*g*. The supernatant was diluted with one volume of water, and four additional volumes of water containing 4 g (dry weight) of calcium phosphate gel (*12*) were added. The gel was collected by centrifugation and washed twice with 200 ml of 0.08*M* potassium phosphate buffer. The enzyme was eluted from the gel with 240 ml of 0.5*M* phosphate buffer. This buffer and all solutions used in succeeding steps contained $10^{-3}M$ dithiothreitol. The protein precipitated from the gel eluate by the addition of $(NH_4)_2SO_4$ to 75 percent saturation was collected by centrifugation (15 minutes at 33,000*g*) and dissolved in 160 ml of 0.05*M* phosphate buffer. Four volumes of water containing 800 mg (dry weight) of alumina C-gamma gel (Sigma) were added, and the gel was eluted with 240 ml of 0.125*M* phosphate buffer. The gel eluate was fractionated by the addition of $(NH_4)_2SO_4$, and the protein precipitating between 30 and 47 percent saturation was dissolved in 190 ml of 0.0085*M* phosphate buffer. The enzyme in this solution was adsorbed on a column (2.4 by 6 cm) of Serva diethylaminoethyl-cellulose (DEAE) (equilibrated with 0.025*M* phosphate buffer) and eluted with successive portions (50 ml) of 0.10, 0.15, and 0.25*M* phosphate buffer. The active fractions were usually eluted by 0.15*M* phosphate buffer. They were pooled, concentrated to 2 to 3 ml by ultrafiltration, dialyzed 18 hours against 0.005*M* tris buffer, *p*H 7.8, and stored on ice. The yield was 6 to 7 mg of protein that catalyzed the transfer of 2 to 4 μmole of phosphate per hour per milligram of protein to f1 histone under standard assay conditions (*13*).

The effect of cyclic AMP on the activity of histone kinase was measured in reaction mixtures (0.5 ml) containing tris buffer, *p*H 7.5, 25 μmole; $MgCl_2$, 2.5 μmole; dithiothreitol, 0.5 μmole; histone or protamine (*14*), 50 μg; and β,γ ^{32}P-labeled adenosine triphosphate (ATP) (*15*), 0.25 μmole. The reactions were initiated by the addition of ^{32}P-ATP, incubated at 37°C, and terminated by the addition of 2 ml of cold 31 percent trichloroacetic acid. Acid-insoluble material was collected on HA Millipore filters and washed with a total of 30 to 35 ml of 25 percent trichloroacetic acid. The filters were placed in 2 ml of 1*N* NaOH containing 0.1 μmole of carrier inorganic phosphate and heated at 100°C for 15 minutes. Phosphate esterified to serine residues is released as inorganic phosphate by this treatment. After being

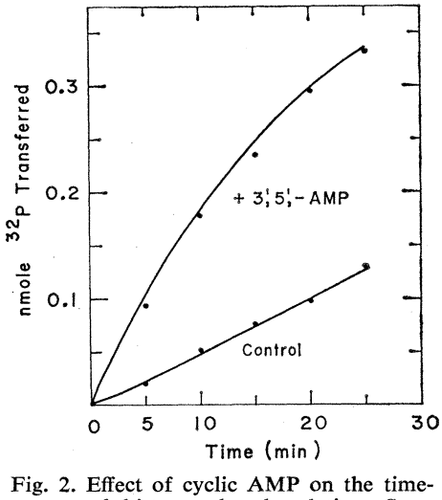

Fig. 2. Effect of cyclic AMP on the time-course of histone phosphorylation. Samples were taken from two large reaction mixtures, and the reactions were terminated at the times indicated. One mixture contained $5 \times 10^{-6}M$ cyclic AMP; the substrate was f1 histone, and the amount of histone kinase per 0.5 ml of reaction mixture was 1.3 μg. The specific activity of the β,γ-ATP phosphate was 6200 count/min per nanomole. The figures on the ordinate indicate the amount of ^{32}P transferred per 0.5 ml of reaction mixture.

Table 1. Stimulation of histone phosphorylation by cyclic AMP. In addition to the components listed in the text, the reaction mixtures contained 0.66 μg of histone kinase and, where indicated, 0.0025 μmole of cyclic AMP (Sigma); the specific activity of the ^{32}P-ATP was 6930 count/min per nanomole of β,γ-phosphate. The incubation time was 5 minutes.

Substrate	^{32}P Transferred (pmole)		Stimulation (%)
	+ Cyclic 3′,5′-AMP	Control	
f1 Histone	124	23	530
f2b Histone	364	92	400
Protamine	62	57	110
None	5	8	

cooled, protein and filter debris were precipitated by addition of 0.5 ml of 4*N* HCl, 1*N* in H_2SO_4, followed by 0.1 ml of 0.1*M* silicotungstic acid, 0.1*N* in H_2SO_4. To 2.0 ml of the supernatant, 0.5 ml of 5 percent ammonium molybdate, 4*N* in H_2SO_4 was added, and the resulting phosphomolybdate complex was extracted with 2.5 ml of isobutanol-benzene, 1:1 (*16*). Portions (2.0 ml) of the extracts were plated and counted in a thin-window GM counter.

The effect of cyclic AMP on the phosphorylation of histones and protamine is presented in Table 1. In other experiments, the increased histone phosphorylation observed under these conditions ranged from four- to sixfold (400 to 600 percent). There was little or no increase in protamine phosphorylation. Half-maximum stimulation of histone phosphorylation is produced by approximately $3 \times 10^{-8}M$ cyclic AMP, and the maximum effect is obtained at $10^{-7}M$ (Fig. 1). The 5′- and mixed 2′- + 3′-monophosphates of adenosine, uridine, guanosine, and cytidine ($10^{-5}M$) did not replace cyclic AMP.

Cyclic AMP affects the initial rate of histone phosphorylation, and this rate remains approximately constant under the conditions of incubation for 10 minutes in both its presence and absence (Fig. 2). The greatest effects of cyclic AMP are obtained at low (less than saturating) concentrations of histone. For example, raising the histone concentration from 0.1 to 1.0 mg/ml increases the rate of phosphorylation in control reactions fivefold; the stimulation produced by cyclic AMP at these two concentrations of histone was 4.6- and 3.3-fold, respectively.

Cyclic AMP does not change the extent of phosphorylation of histones. The maximum amount of phosphate that can be transferred to f1 histone on prolonged incubation with large amounts of enzyme and excess ATP is 33 to 40 nmole/mg, regardless of the presence of cyclic AMP. These data indicate that the site of phosphorylation of f1 histone, which in the absence of cyclic AMP is mainly a single specific serine residue (*17*), is the same in the presence of the cyclic nucleotide.

Huang and Bonner and Allfrey *et al.* (*18*) have shown that histones can inhibit RNA synthesis in cell-free systems. Their findings have caused interest in the possibility that histones may act as repressors of DNA template activity in eukaryotic cells. The additional possibility that acetylation (*19*) or phosphorylation (*20*) of histones may modify DNA-histone interactions and cause derepression of template activity has also been considered. In this context, the observation that cyclic AMP stimulates histone phosphorylation suggests a mechanism for the induction of RNA synthesis by those hormones that cause increases in the concentration of cyclic AMP.

THOMAS A. LANGAN
Charles F. Kettering Research Laboratory, Yellow Springs, Ohio 45387

References and Notes

1. T. A. Langan and L. K. Smith, *Fed. Proc.* **26**, 603 (1967).
2. E. W. Sutherland and T. W. Rall, *Pharmacol. Rev.* **12**, 265 (1960).
3. E. G. Krebs, D. S. Love, G. E. Bratvold, K. A. Trayser, W. L. Meyer, E. H. Fischer, *Biochemistry* **3**, 1022 (1964).
4. R. J. DeLange, R. G. Kemp, W. D. Riley, R. A. Cooper, E. G. Krebs, *J. Biol. Chem.* **243**, 2200 (1968).
5. D. A. Walsh, J. P. Perkins, E. G. Krebs, *ibid.*, p. 3763.
6. G. A. Robison, R. W. Butcher, E. W. Sutherland, *Ann. N.Y. Acad. Sci.* **139**, 703 (1967).
7. J. J. Ferguson, Jr., *J. Biol. Chem.* **238**, 2754 (1963); J. M. Marsh, R. W. Butcher, K. Savard, E. W. Sutherland, *ibid.* **241**, 5436 (1966); D. G. Grahame-Smith, R. W. Butcher, R. L. Ney, E. W. Sutherland, *ibid.* **242**, 5535 (1967); D. Holten and F. T. Kenney, *ibid.*, p. 4372; W. D. Wicks, *Science* **160**, 997 (1968).
8. K. Savard, J. M. Marsh, B. F. Rice, *Recent Progr. Hormone Res.* **21**, 285 (1965); R. V. Farese, *Endocrinology* **78**, 929 (1966); D. Holten and F. T. Kenny, *J. Biol. Chem.* **242**, 4372 (1967).
9. K. Marushige, V. Ling, B. Jergil, G. H. Dixon, *Fed. Proc.* **27**, 336 (1968).
10. I thank Dr. G. H. Dixon for communicating these results prior to publication and Dr. E. G. Krebs for providing a manuscript of the paper quoted in (*5*).
11. Prepared by homogenizing calf liver in a Waring blender with ten volumes of chilled acetone (—15°C) and washing three times with five-volume portions of acetone.
12. Prepared according to Keilin and Hartree [in *Meth. Enzymol.* **1**, 98 (1955)].
13. Histone kinase activity is assayed under the conditions described with the following exceptions: histone concentration, 1 mg/ml; cyclic AMP omitted; final volume, 0.25 ml; incubation time, 20 minutes. For routine assay, acid-insoluble ^{32}P was determined as described in the legend to Fig. 1.
14. Calf thymus histones were prepared according to the methods of E. W. Johns, *Biochem. J.* **92**, 55 (1964). Protamine was prepared from salmon sperm by the method of M. J. Callanan, W. R. Carroll, E. R. Mitchell, *J. Biol. Chem.* **229**, 279 (1957).
15. β,γ^{32}P-Adenosine triphosphate was prepared with a photophosphorylation system adapted from B. Petrack, A. Craston, F. Sheppy, F. Farron [*J. Biol. Chem.* **240**, 906 (1965)] and purified by chromatography on DEAE-cellulose with NH_4HCO_3 buffer, *p*H 8.6.
16. J. B. Martin and D. M. Doty, *Anal. Chem.* **21**, 965 (1949).
17. T. A. Langan, in *Some Regulatory Mechanisms for Protein Synthesis in Mammalian Cells*, A. San Pietro, M. Lamborg, F. T. Kenny, Eds. (Academic Press, New York, in press).
18. R. C. Huang and J. Bonner, *Proc. Nat. Acad. Sci. U.S.* **48**, 1216 (1962); V. G. Allfrey, V. C. Littau, A. E. Mirsky, *ibid.* **49**, 414 (1963).
19. B. G. T. Pogo, A. O. Pogo, V. G. Allfrey, A. E. Mirsky, *ibid.* **59**, 1337 (1968).
20. M. G. Ord and L. A. Stocken, *Biochem. J.* **107**, 403 (1968); W. S. Stevely and L. A. Stocken, *ibid.* **100**, 20C (1966); L. J. Kleinsmith, V. G. Allfrey, A. E. Mirsky, *Proc. Nat. Acad. Sci. U.S.* **55**, 1182 (1966).
21. Contribution No. 317 of the C. F. Kettering Research Laboratory. Supported by NIH grant CA 10278. I thank J. Koontz for technical assistance, and Dr. Waldo Zaugg for supplies of salmon sperm.

26 June 1968; revised 26 August 1968

Hypothalamic Stimulation of Growth Hormone Secretion

Abstract. *Stimulation of the ventromedial hypothalamic nucleus in rats resulted in increased plasma growth hormone levels within 5 minutes, as measured by radioimmunoassay. Stimulation of the cerebral cortex was without effect. These observations confirm previous results involving destructive lesions and establish the ventromedial nucleus as a hypothalamic locus involved in the control of growth hormone secretion.*

Evidence for the hypothalamic control of growth hormone secretion has been based primarily on experiments involving destructive hypothalamic lesions. In most studies, however, large areas of the ventral hypothalamus, including the median eminence, have been destroyed (*1*), thus precluding the specific localization of the site of control of growth hormone secretion. Recent studies from our laboratory (*2*) have demonstrated that destruction of the ventromedial hypothalamic nucleus (VMN) in weanling rats results in impaired growth associated with decreased growth hormone levels in both the pituitary and plasma. Since the median eminence was undamaged in these animals, we suggested that the hypothalamic control of growth hormone secretion resides in the VMN. The present report offers evidence of a different nature to support this hypothesis.

Female Holtzman rats weighing 180 to 220 g were anesthetized with pento-